

## RESEARCH PAPER

# Increased glutamine in leaves of poplar transgenic with pine GS1a caused greater anthranilate synthetase $\alpha$ -subunit (ASA1) transcript and protein abundances: an auxin-related mechanism for enhanced growth in GS transgenics?

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## Abstract

The initial reaction in the pathway leading to the production of indole-3-acetic acid (IAA) in plants is the reaction between chorismate and glutamine to produce anthranilate, catalysed by the enzyme anthranilate synthase (ASA; EC 4.1.3.27). Compared with non-transgenic controls, leaves of transgenic poplar with ectopic expression of the pine cytosolic glutamine synthetase (GS1a; EC 6.3.1.2) produced significantly greater glutamine and significantly enhanced ASA  $\alpha$ -subunit (ASA1) transcript and protein (approximately 130% and 120% higher than in the untransformed controls, respectively). Similarly, tobacco leaves fed with 30 mM glutamine and 2 mM chorismate showed enhanced ASA1 transcript and protein (175% and 90% higher than controls, respectively). Furthermore, free IAA was significantly elevated both in leaves of GS1a transgenic poplar and in tobacco leaves fed with 30 mM glutamine and 2 mM chorismate. These results indicated that enhanced cellular glutamine may account for the enhanced growth in GS transgenic poplars through the regulation of auxin biosynthesis.

**Key words:** Anthranilate synthase, glutamine synthetase, indole acetic acid (IAA), nitrogen assimilation, poplar.

## Introduction

Enhancement of the efficiency by which plants assimilate inorganic nitrogen plays a central role in improving agronomic performance of crop and forest species. The central role of glutamine synthetase (GS; EC 6.3.1.2) in plant nitrogen metabolism has been well-documented (Lam *et al.*, 1995, 1996, for a review). In order to improve the efficiency of nitrogen assimilation, several groups have reported transgenic plants characterized by enhanced expression of glutamine synthetase, including tobacco, lotus, alfalfa, and poplar (Eckes *et al.*, 1989; Hemon *et al.*, 1990; Hirel *et al.*, 1992; Temple *et al.*, 1993; Gallardo *et al.*, 1999; Olivera *et al.*, 2002; Kirby *et al.*, 2006). The results of these studies have been mixed. An alternative approach to enhance nitrogen utilization efficiency has focused on

ectopic expression of bacterial glutamate dehydrogenase in transgenic tobacco and corn (Ameziane *et al.*, 2000; Mungur *et al.*, 2005) resulting in the ammonium incorporation into glutamate. Results from these studies have shown enhanced vegetative growth and drought resistance.

In addition to its role in ammonium assimilation, glutamine is directly involved in the initial reaction in the pathway leading to *de novo* purine biosynthesis (Smith and Atkins, 2002). The intermediate products of this pathway, including AMP and GMP, provide purine bases for nucleic acid biosyntheses, for the synthesis of a number of essential coenzymes (NAD, NADP, FAD, and coenzyme A), and for the biosynthesis of signalling molecules, such as cAMP.

The initial step in the pathway for both tryptophan-dependent and tryptophan-independent pathways for the biosynthesis of indole-3-acetic acid (IAA) is the transfer of the  $\alpha$ -amino group of glutamine to chorismate producing anthranilate. This reaction is catalysed by anthranilate synthase (ASA; EC 4.1.3.27) (Crozier *et al.*, 2000; Cho *et al.*, 2000). Plant ASA holoenzymes are tetramers consisting of two  $\alpha$ - and two  $\beta$ -subunits encoded by separate nuclear genes. The  $\alpha$  and  $\beta$  subunits of ASA are initially synthesized in the cytosol as precursor proteins with plastid-targeting transit peptides (Zalkin *et al.*, 1984; Crawford, 1989; Radwanski and Last, 1995). The functional holoenzyme is then assembled in the plastids (Poulsen *et al.*, 1993; Bohlmann *et al.*, 1995; Romero and Roberts, 1996). The ASA  $\beta$ -subunit (ASB1) is an aminotransferase that cleaves glutamine releasing the  $\alpha$ -amino group, which is transferred by the ASA  $\alpha$ -subunit (ASA1) to chorismate producing anthranilate (Zhang *et al.*, 2001; Inaba *et al.*, 2007). Analyses of the nucleotide sequences coding for ASA  $\alpha$ -subunits (*ASA1*) from rice, *Arabidopsis*, *Ruta graveolens*, and tobacco reveal a high degree of sequence homology (Tozawa *et al.*, 2001; Niyogi and Fink, 1992; Bohlmann *et al.*, 1996). Furthermore, anthranilate synthase has been implicated in secondary metabolism, including alkaloid biosynthesis in *Ruta graveolens* and rice (Bohlmann *et al.*, 1996; Tozawa *et al.*, 2001).

Recent reports have shown that the increase of free IAA in transgenic potato lines expressing the rice anthranilate synthase  $\alpha$ -subunit gene *OAS1D* did not alter the phenotypes of the transgenics (Matsuda and Yamada, 2005). However, it has been proposed that the increases in IAA biosynthesis in plants might undergo counteraction by metabolic conversion mechanisms that maintain IAA homeostasis (Normanly, 1997; Ljung *et al.*, 2002; Matsuda and Yamada, 2005).

Transgenic poplar with ectopic expression of the pine cytosolic GS1a exhibit enhanced GS activity, enhanced free glutamine in leaves, and enhanced vegetative growth (Gallardo *et al.*, 1999; Fu *et al.*, 2003; Man *et al.*, 2005; Kirby *et al.*, 2006). These results prompted us to examine the mechanism for enhanced vegetative growth in GS poplars by studying the relationship between enhanced glutamine synthesis, ASA gene expression, and auxin homeostasis.

## Materials and methods

### Plant materials

Hybrid poplar (*Populus tremula* L.  $\times$  *P. alba* L., clone 'INRA 7171-B4'), was used in this study. This clone was selected because of uniform growth and ease of transformation by *Agrobacterium* (Leplé *et al.*, 1992). Transgenic lines with ectopic expression of pine glutamine synthetase GS1a (Gallardo *et al.*, 1999) and non-transgenic controls were grown in sterile potting mix in 15 cm diameter pots (MetroMix 200, Scotts Co., Marysville, Ohio, USA) in a growth chamber (16 h photoperiod; 295–330  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; 24 °C). All plants, including transgenic lines and non-transgenic controls, were clonally propagated from rooted stem cuttings.

Plants were supplied with 30 ml of 1 mM ammonium and 3 mM nitrate applied to each pot twice weekly (Johnson *et al.*, 1957). Uniform experimental plants approximately 60 cm tall of high- and low-performing transgenic lines (lines 4-29 and 12-9, respectively) and non-transformed controls were randomly selected. In order to account for minor within-clone differences based on growth conditions, eight plants for each transgenic line and eight non-transgenic controls were used. All plants were numbered and their locations in the growth chamber were assigned randomly. Tobacco plants (*Nicotiana tabacum* L., line SR-1) were also grown in potting mix in a growth chamber under the same growth conditions described above.

For biochemical and molecular analyses, fully expanded mature leaves (leaf ranks 7–10) were taken from four plants of the two transgenic poplar lines and controls. Leaves were cut into 0.5  $\times$  0.5 cm segments, weighed, frozen in liquid nitrogen, and stored at –86 °C (Man *et al.*, 2005). Each experiment was performed at least twice (in duplicate).

### Amino acid analysis

The content of free amino acids in leaves was determined using high performance liquid chromatography (HPLC). Leaf discs (approximately 200 mg) from GS1a transgenic and control plants were placed in Eppendorf tubes containing 800  $\mu\text{l}$  of 5% (v/v) ice-cold perchloric acid. Samples were frozen at –20 °C. The frozen samples were thawed and refrozen three times, and centrifuged at 13 500 g for 10 min. Amino acids were analysed by HPLC according to the method of Minocha and Long (2004). Four replicate determinations were run for each treatment. External standards consisted of a mix of 23 amino acids. The detectable total free amino acid pool of poplar leaf tissue consisted mainly of aspartic acid, glutamic acid, glutamine, serine, arginine, cysteine, glycine, proline,  $\gamma$ -aminobutyric acid (GABA), valine, methionine, isoleucine, leucine, tryptophan, phenylalanine, ornithine, lysine, and histidine. Threonine, asparagine, tyrosine, cysteine, and hydroxyproline could not be quantified using this method (Minocha and Long, 2004).

### Tobacco leaf feeding

Tobacco leaves were supplied with exogenous amino acids as previously described (Tsuno *et al.*, 1983). Four fully-expanded tobacco leaves from four different tobacco plants were used for each treatment. One half of each leaf was cut from the middle vein longitudinally and placed petiole down into a beaker containing an aqueous test solution of amino acids (30 mM, pH 7.0). The remaining half of each leaf was placed in a beaker containing MilliQ water (control). Immediately after being placed in the beakers, approximately 1 cm of each petiole was excised below the surface of the solution in order to maintain the intact water column of the cut leaves and to facilitate uniform uptake of the experimental solution. Experimental leaves were maintained in the growth chamber for 3 h (conditions defined above) after which they were blotted dry, cut into 0.4  $\times$  0.4 cm segments, frozen in liquid nitrogen, and stored at –80 °C.

### RNA extraction and expression analysis

A consensus primer pair specific to the ASA  $\alpha$ -subunit was designed using the multiple alignment system of 'Vector NTI Suite, Version 6.0' (InforMax Inc., Bethesda, MD, USA). The pair (sense primer 5'-AATGATGTTGGAAAGGTGTC-3'; antisense primer, 5'-ACCTTTGGTGCTCCCAAC-3') were used to detect an expected 184 bp PCR fragment of the ASA  $\alpha$ -subunit. Primer pairs were aligned with available DNA sequences at the NCBI database using the 'Gapped BLAST and PSI-BLAST 2.0' (Altschul *et al.*, 1997). Results indicated that the identified primer pair is specific to plant anthranilate synthase (ASA)  $\alpha$ -subunit. Furthermore, in order to ensure that no identical sequences

specific to the ASA  $\alpha$ -subunit exist in the transformation vector used to construct the pine GS1a transformed poplar, the primer sequences were also aligned with the transformation vector sequences using 'PAIRWISEBLAST'. No identical or similar sequences were found.

Total RNA was isolated from poplar and tobacco leaves according to Chang *et al.* (1993) with modifications. After LiCl precipitation, the DNA-free kit (Ambion, Austin, TX, USA) was used to remove genomic DNA residues from total RNA according to the manufacturer's protocol.

An iScriptSelect cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) was used in the RT reaction. Oligo (dT) primers were used to produce the entire length of cDNAs from mRNAs. The detailed RT reaction procedures were followed as recommended by the manufacturer. RT-PCR was performed using the 18S rRNA gene (Ambion, Austin, TX, USA) as an internal standard (324 bp amplicon) according to manufacturer's protocol. The resulting RT-PCR products were analysed on 1.5% (w/v) agarose gels using standard procedures.

Quantitative real-time PCR assays (q-PCR) were performed using a LightCycler 480<sup>®</sup> instrument (Roche Diagnostics, Indianapolis, IN, USA) with polyubiquitin (*UBQ11*) as the reference gene (Brunner *et al.*, 2004). All reactions were carried out in a 20  $\mu$ l final volume containing 8  $\mu$ l of 1/15 diluted cDNA sample, 1  $\mu$ l of 10  $\mu$ M of each primer (0.5  $\mu$ M final concentration), 10  $\mu$ l 2 $\times$  LightCycler<sup>®</sup> 480 SYBR Green I MasterMix. For each q-PCR run, reactions were done in triplicate. Two independent q-PCR runs gave identical results. The conditions for q-PCR were as follows: 95  $^{\circ}$ C for 5 min followed by 45 thermal cycles of 95  $^{\circ}$ C for 10 s, 60  $^{\circ}$ C for 20 s, 72  $^{\circ}$ C for 20 s, with a ramping rate of 4.4, 2.2, and 2.2  $^{\circ}$ C s<sup>-1</sup>, respectively. SYBR specific fluorescence (483–533 nm) at the end of each cycle was measured and recorded via CCD camera and software. Melting curve analyses to verify sequence specificity were run at of 95  $^{\circ}$ C for 5 s (4.4  $^{\circ}$ C s<sup>-1</sup>), 70  $^{\circ}$ C for 1 min (2.2  $^{\circ}$ C s<sup>-1</sup>), 95  $^{\circ}$ C (0.11  $^{\circ}$ C s<sup>-1</sup>). Fluorescence change profiles of thermal dynamic dissociation of DNA were recorded in the last heating step through the continuous data acquisition process (5 acquisitions s<sup>-1</sup>). The relative expression ratio of target mRNAs was calculated using the LightCycler<sup>®</sup> 480 Software release 1.5.0 (Roche Diagnostics, Indianapolis, IN, USA). Relative standard curves were generated using 1/10, 1/20, and 1/40 dilutions of cDNA, respectively.

#### Electrophoresis and immunoblot analysis

Total soluble proteins were extracted from poplar leaves as described previously (Gallardo *et al.*, 1999). Protein content was estimated (Bradford, 1976) using bovine serum albumin as the standard. Proteins were separated by SDS-PAGE (10% v/v acrylamide) using the discontinuous buffer system of Laemmli (1970). Equal amounts of protein (25  $\mu$ g) were loaded in each lane. Gels were stained with Coomassie blue.

Following electrophoresis, proteins were electrotransferred on to nitrocellulose membranes (0.45  $\mu$ m pore size, Whatman PROTRAN BA 85; Dassel, Germany) using the Bio-Rad wet blotting system at 4  $^{\circ}$ C with constant current (40 mA) overnight. After transfer, the membranes were incubated with polyclonal primary rabbit antibodies prepared against *Arabidopsis* ASA1 (anthranilate synthase  $\alpha$ -subunit 1, 595 amino acids, approximately 66 kDa) (Niyogi and Fink, 1992; Bernasconi *et al.*, 1994; Rutherford *et al.*, 1998) (1:10 000 dilution) (antibodies were generously provided by Professor Jack Widholm, University of Illinois). The Vectastain ABC-AmP kit (Vector Laboratories, Burlingame, CA, USA) with biotinylated secondary antibody (alkaline phosphatase conjugated, goat-anti-rabbit IgG) was used for immunodetection according to the manufacturer's protocols. Scanned images of bands were quantified with ImageJ 1.42q (National Institute of Health, Bethesda, MD, USA) according to established protocols (Tarlton and Knight, 1996; Vierck *et al.*,

2001; Mousli *et al.*, 2003). Means of 4–8 measurements of digital counts of luminosity of each band were used to calculate relative band densities for comparisons of the different treatments.

#### Free IAA determination

Free IAA contents were measured using GS-MS according to the procedure of Muller (Mueller and Weiler, 2000; Pollmann *et al.*, 2009). Final values are reported as means of 12 measurements from triplicate feeding treatments.

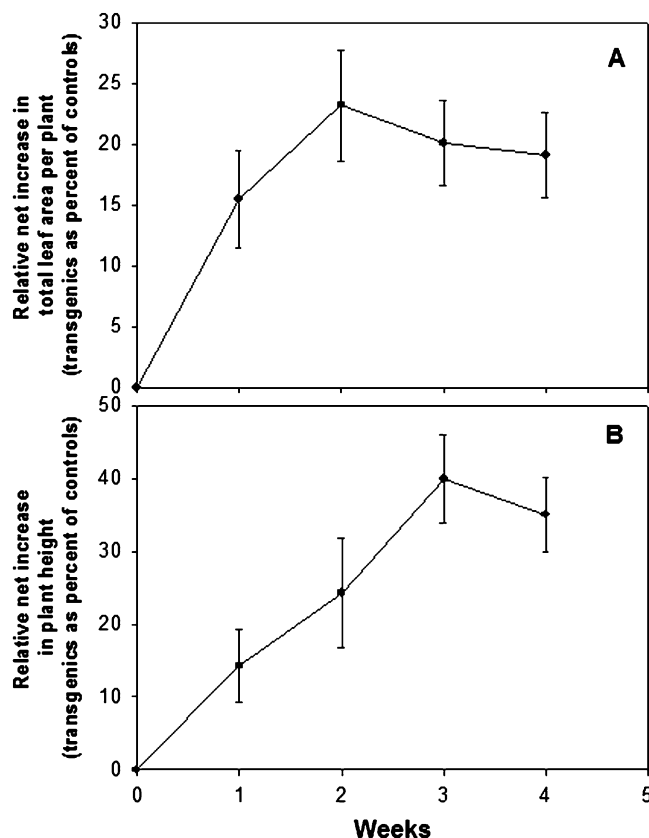
#### Statistical analysis

The *t* test for all treatments was carried out using SigmaPlot 11.0 (Systat Inc., San Jose, CA). In addition, each pair of amino acid feeding treatments was compared with each other. Significance was accepted at  $P \leq 0.05$  and  $P \leq 0.01$ .

## Results

### *Vegetative growth and leaf GS activities of transgenic poplar*

Transgenic poplar plants expressing the pine cytosolic GS1a exhibited significant increases in growth over non-transgenic controls during 4 weeks growth (Fig. 1). Increases were shown for relative total leaf area (Fig. 1A)



**Fig. 1.** Growth parameters of GS1a transgenic poplar and controls grown for 5 weeks under 10 mM nitrate conditions, as described. Each value represents the mean of four replicate determinations. (A) Relative net increase in total leaf area per plant as a percentage of the controls; (B) relative net increase in plant height as a percentage of the controls.



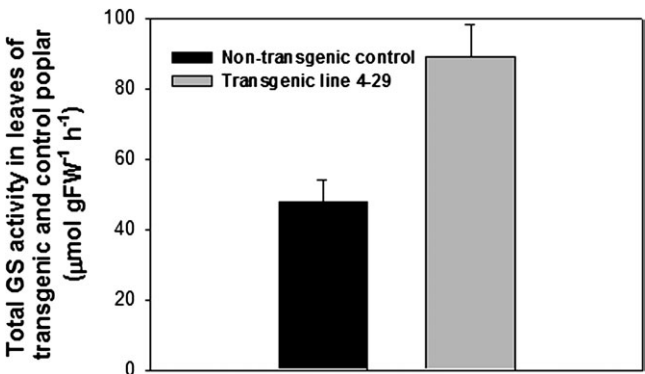
and relative plant height (Fig. 1B). Transgenics achieved maximum increases in relative height growth and relative leaf area growth after 3 weeks. Furthermore, after 4 weeks, GS activity (means of 4–6 plants) in leaves of transgenic poplar were significantly enhanced ( $93.1 \mu\text{mol g}^{-1} \text{FW h}^{-1}$ ) compared with the amounts in leaves of non-transgenic controls ( $54.2 \mu\text{mol g}^{-1} \text{FW h}^{-1}$ ) (Fig. 2). These results are consistent with previously published results (Gallardo *et al.*, 1999; Fu *et al.*, 2003).

*Analysis of free amino acids in leaves of GS1a transgenic and control poplar, and in tobacco leaves supplied with glutamine and chorismate*

In order to assess the consequences of GS overexpression on nitrogen homeostasis, glutamine and total free amino acid contents in leaves of transgenic and control poplars were measured (Table 1). Free glutamine contents in GS transgenic leaves were significantly higher than contents in non-transgenic controls ( $1381.4$  versus  $418.78 \text{ nmol g}^{-1} \text{FW}$ ;  $229.9\%$ ). Furthermore, glutamate, tryptophan, and GABA were also significantly enhanced in the GS1a transgenic poplars. Although aspartate contents in transgenics

were higher, the differences were not significant. Contents of total free amino acid were significantly enhanced in GS poplar leaves (approximately 20% higher than for non-transgenic controls) (Table 1).

To confirm the results obtained with GS1a transgenic poplar, a leaf feeding technique was used to supply tobacco leaves with exogenous glutamine, as previously described by Tsuno *et al.* (1983). The contents of free amino acids, including glutamine, glutamate, phenylalanine, tryptophan, aspartate,  $\gamma$ -amino butyric acid (GABA), and arginine in tobacco leaves supplied with 30 mM glutamine and 2 mM chorismate are reported in Table 2. Free glutamine in tobacco leaves supplied with 30 mM glutamine was significantly enhanced over the paired control supplied with water alone ( $97.6 \text{ mmol g}^{-1} \text{FW}$  versus  $0.922 \text{ mmol g}^{-1} \text{FW}$ ;  $P < 0.01$ ). Glutamine contents in tobacco leaves supplied with 30 mM L-glutamine and 2 mM chorismate were also increased over the contents in leaves supplied with water alone ( $56.9 \text{ mmol g}^{-1} \text{FW}$  versus  $0.98 \text{ mmol g}^{-1} \text{FW}$ ;  $P < 0.01$ ). In tobacco leaves supplied with 2 mM chorismate alone, free glutamine showed low values with little difference from the paired control provided with water alone ( $0.99 \text{ mmol g}^{-1} \text{FW}$  versus  $1.16 \text{ mmol g}^{-1} \text{FW}$ ). Furthermore, glutamate, aspartate, and GABA were elevated in leaves supplied with glutamine, either with or without chorismate. Phenylalanine was enhanced in leaves supplied with chorismate, either in the presence of or absence of glutamine (Table 2).



**Fig. 2.** Total GS activity in leaves of GS1a transgenic and control poplar grown under 10 mM nitrate conditions. Each value is the mean of four replicate determinations; values are reported as  $\mu\text{mol g}^{-1} \text{FW h}^{-1}$ .

**Table 1.** Free amino acids in leaves of transgenic and control poplar grown under high (10 mM) nitrogen conditions

Values are reported as  $\text{nmol g}^{-1} \text{FW}$  (\* represents significance at  $P < 0.05$  level). Each value is the mean of four replicate determinations of six pooled transgenic lines ( $\pm \text{SE}$  as indicated).

	Content in non-transgenic controls ( $\text{nmol g}^{-1} \text{FW}$ )	Content in G1a transgenics ( $\text{nmol g}^{-1} \text{FW}$ )	Per cent increase (G1a transgenics versus non-transgenic controls)
Gln	$418.8 \pm 38.3$	$1381.4 \pm 209.6^*$	229.9%
Glu	$1764.2 \pm 217.3$	$2656.5 \pm 293.9^*$	50.6%
Asp	$1187.6 \pm 153.2$	$1567.4 \pm 183.0$	31.9%
Trp	$46.7 \pm 1.8$	$89.9 \pm 6.0^*$	92.5%
GABA	$316.9 \pm 26.2$	$959.2 \pm 87.0^*$	202.6%
Total amino acids	$6468.2 \pm 750.1$	$7731.3 \pm 930.4^*$	19.5%

*ASA  $\alpha$ -subunit transcript in leaves of GS1a and control poplars and in tobacco leaves supplied with glutamine and chorismate*

Analysis of RT-PCR products on agarose gels showed that the ASA  $\alpha$ -subunit transcripts in GS1a transgenic poplar was enhanced (Fig. 3B) when compared with both the non-transgenic control (Fig. 3A) and the low-performing transgenic line 4-18 (Fig. 3C). The ASA  $\alpha$ -subunit transcript in the high-performing line 4-29 was approximately 60% greater than in the non-transgenic control, as determined by image analysis (Image J software, NIH).

In the related paired leaf experiment, tobacco leaves provided with 30 mM glutamine showed an enhancement of ASA  $\alpha$ -subunit transcriptss when compared with its corresponding paired water control (Fig. 4, lanes A and B). Tobacco leaves were also provided with 30 mM glutamine plus 2 mM chorismate, both substrates for ASA. Assessment of the ASA  $\alpha$ -subunit transcript on gels (Fig. 4, lane D) showed the same trend as that observed with 30 mM glutamine alone. Supplying leaves with 2 mM chorismate alone showed no clear effect on the ASA  $\alpha$ -subunit transcript compared with the corresponding control (Fig. 4, lane C).

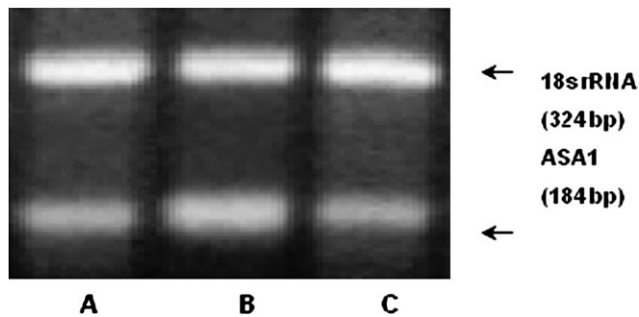
*q-PCR analysis of ASA  $\alpha$ -subunit transcript*

Quantitative RT-PCR (q-PCR) analysis of ASA  $\alpha$ -subunit transcripts in leaves of GS1a transgenic poplar are

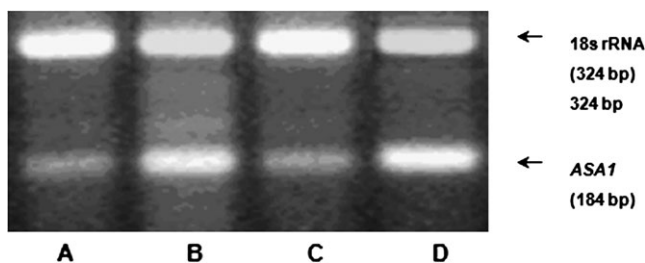
**Table 2.** Free amino acids in tobacco leaves fed with 30 mM glutamine and 2 mM chorismate, as indicated

Each value represents the mean of three replicate determinations of four pooled half leaves,  $\pm$ SD as indicated. Values are reported as nmol g<sup>-1</sup> FW). \*\* Indicates a within-treatment significance of  $P < 0.01$ .

Analysis	Treatment		Glutamine plus chorismate			
	Glutamine alone 30 mM Gln	0 Gln (paired leaves)	30 mM Gln plus 2 mM chorismate	0 Gln/0 chorismate (paired leaves)	Chorismate alone 2 mM chorismate	0 chorismate (paired leaves)
In	97642.1** $\pm$ 4426.7	922.9 $\pm$ 89.2	56900.7** $\pm$ 1655.6	979.2 $\pm$ 58.04	987.3 $\pm$ 84.3	1157.0 $\pm$ 49.7
Glu	10905.7** $\pm$ 242.0	2771.3 $\pm$ 302.3	7395.0** $\pm$ 135.6	2472.4 $\pm$ 152.1	1429.9 $\pm$ 57.2	2276.5** $\pm$ 230.1
Phe	135.4 $\pm$ 5.8	297.9** $\pm$ 12.7	560.7** $\pm$ 29.3	340.1 $\pm$ 15.6	2001.9** $\pm$ 254.3	284.7 $\pm$ 9.8
Trp	1294.2 $\pm$ 73.4	1043.0 $\pm$ 50.9	1089.1 $\pm$ 23.3	1026.9 $\pm$ 123.7	1362.1 $\pm$ 127.5	1122.4 $\pm$ 67.0
Asp	2184.4** $\pm$ 32.1	1069.8 $\pm$ 28.4	1617.0** $\pm$ 58.6	921.0 $\pm$ 64.7	695.3 $\pm$ 52.4	1067.6 $\pm$ 9.0
GABA	5992.6** $\pm$ 536.0	4532.6 $\pm$ 524.9	4150.1 $\pm$ 166.0	3956.4 $\pm$ 496.6	2988.1 $\pm$ 335.0	4046.5** $\pm$ 376.2
Arg	461.7 $\pm$ 28.3	370.3 $\pm$ 81.4	486.1 $\pm$ 29.1	617.8 $\pm$ 36.4	387.3 $\pm$ 85.8	540.1 $\pm$ 46.2

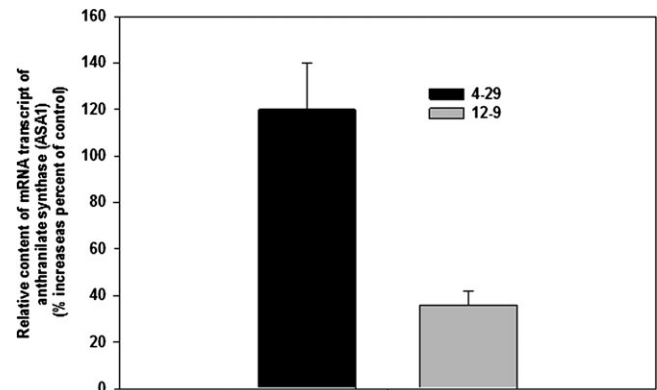


**Fig. 3.** Anthranilate synthase  $\alpha$ -subunit (ASA1) transcript, as detected by RT-PCR, in leaves of (A) non-transgenic poplar; (B) high-performance GS1a transgenic line 4-29; and (C) low-performance GS1a transgenic line 4-18. ASA1 transcript in line 4-29 was approximately 60% greater than in the non-transgenic control, as determined by image analysis (Image J software, NIH).



**Fig. 4.** Anthranilate synthase  $\alpha$ -subunit (ASA1) transcript, as detected by RT-PCR, in paired tobacco leaves, as described, provided with (A) water alone; (B) 30 mM glutamine; (C) 2 mM chorismate; and (D) 30 mM glutamine plus 2 mM chorismate.

presented in Fig. 5. Transcripts are presented as relative content as a percentage increase versus the non-transgenic control. For the high-performing GS transgenic line (4-29), the ASA  $\alpha$ -subunit transcript exceeded the non-transgenic control by approximately 118% ( $P < 0.01$ ). The ASA  $\alpha$ -subunit transcript was also analysed in a second GS1a transgenic line (12-9) characterized by improved growth over the non-transgenic control, but with growth less than that of line 4-29 (Fu *et al.*, 2001). The ASA  $\alpha$ -subunit tran-



**Fig. 5.** Quantitative assessment (q-PCR) of anthranilate synthase  $\alpha$ -subunit (ASA1) transcript in leaves of GS1a poplar compared with non-transgenic controls. Relative contents (per cent increase in ASA1 transcript over non-transgenic controls) are reported for the high performing GS1a transgenic line (4-29) and the lower performing GS1a transgenic line 12-9. Relative contents are reported as averages of two triplicate determinations.

script in leaves of this second transgenic line was also enhanced over the non-transgenic control (38%;  $P < 0.01$ ) (Fig. 5).

q-PCR analysis was also used to assess the ASA  $\alpha$ -subunit transcript in the corresponding tobacco experiment (Fig. 6). In tobacco leaves fed with 30 mM glutamine, the ASA  $\alpha$ -subunit transcript was approximately 175% ( $P < 0.01$ ) greater than the paired control fed with water alone. Tobacco leaves provided with 30 mM glutamine and 2 mM chorismate also showed significant increases in the ASA  $\alpha$ -subunit transcript (approximately 195%;  $P < 0.01$ ) over the paired control. Feeding tobacco leaves with 2 mM chorismate alone showed only a slight increase in the ASA  $\alpha$ -subunit transcript versus the paired control (not significant).

#### Immunoblot analysis of ASA $\alpha$ -subunit

The ASA  $\alpha$ -subunit polypeptide (66 kDa) was detected in immunoblots of protein extracts of both poplar and tobacco leaves (Fig. 7A, B). Quantitative estimates of the

ASA  $\alpha$ -subunit polypeptide in immunoblots (ImageJ Software) showed that the ASA  $\alpha$ -subunit was enhanced in both leaves of the GS1a transgenic poplar (line 4-29) (approximately 120% versus the non-transgenic control) and in tobacco leaves fed with 30 mM glutamine (approximately 90% higher than in the paired controls).

### IAA analysis

Quantitative determinations of free IAA in leaves of GS1a transgenic poplar and in leaves of tobacco fed with 30 mM glutamine are presented in Table 3. IAA in the high-performing GS1a transgenic poplar (line 4-29) averaged 32.15% greater than in the non-transgenic control (116.3 versus 88.0 pmol g<sup>-1</sup> FW;  $P < 0.05$ ). IAA in the moderately performing poplar GS1a transgenic line (12-9) was not statistically different from the non-transgenic control (91.33 versus 88.0 pmol g<sup>-1</sup> FW). In the related tobacco leaf experiment, leaves provided with 30 mM glutamine showed increased IAA contents compared with the control (140.08 versus 112.92 pmol g<sup>-1</sup> FW), however, this increase was not shown to be significant. IAA in tobacco leaves supplied with 30 mM glutamine and 2 mM chorismate was significantly greater than in the paired control (109.83 versus 77.67; 41.4%;  $P < 0.05$ ). Interestingly, IAA contents

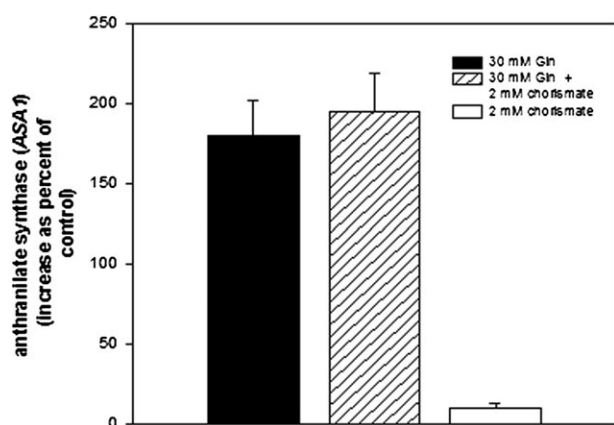
of tobacco leaves supplied with 2 mM chorismate alone were also significantly enhanced over the paired control (143.58 versus 97.0; 48.0%;  $P < 0.001$ ).

## Discussion

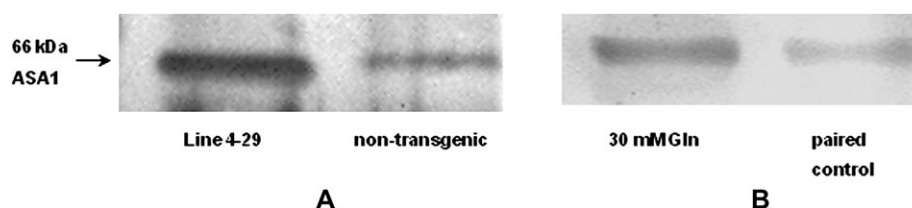
Transgenic poplar characterized by ectopic expression of the pine cytosolic glutamine synthetase (GS1a) display improved growth characteristics when compared with non-transgenic controls. These include enhanced growth in both greenhouse studies (Gallardo *et al.*, 1999; Fu *et al.*, 2003) and field studies (Jing *et al.*, 2004), enhanced nitrogen use efficiency (Man *et al.*, 2005), and enhanced resistance to drought stress (El-Khatib *et al.*, 2004). The present work addresses the hypothesis that enhanced glutamine production may be correlated with alterations in auxin homeostasis.

Analysis of amino acids in leaves of poplar GS transgenics and non-transgenic controls, and in tobacco leaves provided with glutamine and their paired controls produces an insight into how nitrogen homeostasis can be altered as a result of enhanced glutamine production (Tables 1, 2). In order to account for minor within-clone differences based on growth conditions, eight plants for each transgenic line and eight non-transgenic controls were included in the study. Free glutamine contents were significantly higher in transgenic leaves and glutamate, GABA, and tryptophan were also significantly enhanced. These results demonstrate significant alterations in the amino acid homeostasis and metabolism in poplar GS1a transgenics. Similar results were observed in tobacco leaves provided with glutamine and glutamine plus chorismate (Table 2). These shifts in amino acid homeostasis resulting from enhanced glutamine production are directly correlated with and reflect the enhanced growth and photosynthesis capacities of GS1a transgenic poplar (Fu *et al.*, 2003; El-Khatib *et al.*, 2004; Jing *et al.*, 2004; Kirby *et al.*, 2006). Alteration in additional metabolites, including trehalose, an important carbon signal (Paul *et al.*, 2008) could also be anticipated.

In order to address the mechanism(s) for enhanced growth in GS poplars, the relationship between glutamine production in the leaves of GS1a transgenic poplar and the pathway for the production of IAA was studied. Compared with non-transgenic controls, leaves of GS poplars display enhanced expression of the  $\alpha$ -subunit of anthranilate



**Fig. 6.** Quantitative assessment (q-PCR) of anthranilate synthase  $\alpha$ -subunit (ASA1) transcript in leaves of tobacco supplied with (1) 30 mM glutamine; and (2) 30 mM glutamine plus 2 mM chorismate; 2 mM chorismate. Relative contents are calculated as the percentage increase in ASA1 transcript over the pairing controls. Values are reported as averages of two triplicate determinations.



**Fig. 7.** Immunoblot detection anthranilate synthase  $\alpha$ -subunit (ASA1) (66 kDa). (A) Immunoblot from leaf extracts of high performing GS1a transgenic poplar line 4-29 and non-transgenic control; (B) immunoblot of extracts from tobacco leaves provided with 30 mM glutamine and the pairing control.

**Table 3.** Free IAA in leaves of GS1a transgenic poplar lines 4-29 (high performing) and 12-9 (low performing), and in tobacco leaves supplied with 30 mM glutamine and 2 mM chorismate

IAA is reported as pmol g<sup>-1</sup> FW. Values for IAA in poplar leaves represent the means of four replicate determinations ±SD. Tobacco leaves were supplied with glutamine and chorismate, as described with paired controls supplied with water alone. Values for IAA in tobacco leaves represent the means of 12 replicate determinations of four pooled half leaves, ±SD as indicated. Significance levels are as indicated. For per cent increases: \**P* < 0.05; \*\**P* < 0.05; \*\*\**P* < 0.001.

	Poplar leaves			Tobacco leaves					
	Non-transgenic control	GS1a line 4-29	GS1a line 12-9	30 mM Gln	Paired control	30 mM Gln plus 2 mM chorismate	Paired control	2 mM chorismate	Paired control
IAA content (pmol g <sup>-1</sup> FW)	88.0 ±4.96	116.29 ±22.64	91.33 ±10.15	140.08 ±29.07	112.92 ±25.65	109.83 ±37.73	77.67 ±18.9	143.58 ±36.10	97.0 ±22.99
IAA (Per cent increase)		32.15%*	3.80%	24.05%		41.41%**		48.02%***	

synthase (ASA1) transcript and protein (Figs 3, 5, 7). Furthermore, GC/MS analysis of free IAA in poplar leaves revealed that GS poplars have significantly greater free IAA than the non-transgenic control (Table 3). Free IAA has been reported to represent only approximately 1% of the total auxin pool, the remainder being present in sugar and amino acid conjugates of IAA (Park *et al.*, 2001; Pollman *et al.*, 2002; Benjamins and Scheres, 2008). Thus, the IAA values reported here probably represent a small fraction of the total auxin pool.

In order confirm the observed relationship between glutamine and IAA, in a related experiment paired tobacco leaves were provided with free glutamine and chorismate, the precursors to anthranilate. The SR1 line of *N. tabacum* used in this study is characterized by a short life cycle and compact growth habit. Compared with single leaf paired controls supplied with water alone, tobacco leaves supplied with glutamine and chorismate showed significant differences in factors leading to ASA-mediated IAA production including enhanced cellular glutamine, enhanced ASA  $\alpha$ -subunit transcript, enhanced ASA  $\alpha$ -subunit protein, and enhanced free IAA. An anomaly in this study is the increase in IAA observed in tobacco leaves provided with 2 mM chorismate alone. This may indicate that, although IAA was enhanced by glutamine treatment, chorismate availability may be rate-limiting for IAA production in tobacco. Nonetheless, the tobacco study provides confirmation of the relationship between free glutamine and IAA production observed in leaves of GS poplars.

Recent work has pointed to the central role of anthranilate synthase in both the regulation of IAA biosynthesis and in hormone cross-talk. In roots of *Arabidopsis*, a link between ethylene and IAA has been demonstrated through the characterization of two ethylene-insensitive mutants (Stepanova *et al.*, 2005, 2008). This work has shown that ethylene stimulates the auxin biosynthetic pathway via the regulation of ASA expression. Several genes required for IAA synthesis are under transcriptional activation by ethylene and ACC, including WEI2/ASA1 (anthranilate synthase  $\alpha$ 1) and WEI7/ASB1 (anthranilate synthase  $\beta$ 1). Furthermore, the tryptophan-deficient dwarf (*tdd1*) in

*Arabidopsis*, which codes for the ASA  $\beta$ -subunit (ASAB1), was found to have low IAA and significant alterations in floral and embryonic development (Sazuka *et al.*, 2009), thus demonstrating a clear relationship between the transcriptional regulation of ASA and IAA production. These results implicate glutamine in the regulation of free IAA in leaves via the transcriptional regulation of ASA. IAA plays a central role in plant growth and development (see Chandler, 2009; Normanly, 2010; Zhao, 2010, for recent reviews). GS transgenic poplars display enhanced growth characteristics, thus the enhanced growth is, at least in part, correlated with the role that glutamine plays in regulating IAA production.

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